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<p>(21) International Application Number: PCT/US95/03216 (22) International Filing Date: 15 March 1995 (15.03.95)  (71) Applicant (for all designated States except US): HUMAN GENOME SCIENCES, INC. [US/US]; 9410 Key West Avenue, Rockville, MD 20850-3338 (US).  (72) Inventors; and (75) Inventors/Applicants (for US only): GREENE, John, M. [US/US]; 872 Diamond Drive, Gaithersburg, MD 20878 (US). FLEISCHMANN, Robert, D. [US/US]; 470 Tschiffely Square Road, Gaithersburg, MD 20878 (US).  (74) Agents: OLSTEIN, Elliot, M.; Carella, Byrne, Bain, Gilfillan, Cecchi, Stewart &amp; Olstein, 6 Becker Farm Road, Roseland, NJ 07068 (US) et al.</p>		<p>(81) Designated States: AU, CA, CN, JP, KR, MX, NZ, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> With international search report.</p>
<p>(54) Title: HUMAN TUMOR NECROSIS FACTOR RECEPTOR (57) Abstract  A human TNF receptor and DNA (RNA) encoding such receptor and a procedure for producing such receptor by recombinant techniques is disclosed. Also disclosed are methods for utilizing such receptor for screening for antagonists and agonists to the receptor and for ligands for the receptor. Also disclosed are methods for utilizing such agonists to inhibit the growth of tumors, to stimulate cellular differentiation, to mediate the immune response and anti-viral response, to regulate growth and provide resistance to certain infections. The use of the antagonists as a therapeutic to treat autoimmune diseases, inflammation, septic shock, to inhibit graft-host reactions, and to prevent apoptosis is also disclosed. Also disclosed are diagnostic methods for detecting mutations in the nucleic acid sequence encoding the receptor and for detecting altered levels of the soluble receptor in a sample derived from a host.</p>		

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### HUMAN TUMOR NECROSIS FACTOR RECEPTOR

This invention relates to newly identified polynucleotides, polypeptides encoded by such polynucleotides, the use of such polynucleotides and polypeptides, as well as the production of such polynucleotides and polypeptides. The polypeptide of the present invention has been putatively identified as a Tumor Necrosis Factor receptor, and more particularly as a type 2 Tumor Necrosis Factor Receptor. The polypeptide of the present invention will hereinafter be referred to as "TNF receptor". The invention also relates to inhibiting the receptor.

Human tumor necrosis factors  $\alpha$  (TNF- $\alpha$ ) and  $\beta$  (TNF- $\beta$  or lymphotoxin) are related members of a broad class of polypeptide mediators, which includes the interferons, interleukins and growth factors, collectively called cytokines (Beutler, B. and Cerami, A., Annu. Rev. Immunol., 7:625-655 (1989)).

Tumor necrosis factor (TNF- $\alpha$  and TNF- $\beta$ ) was originally discovered as a result of its anti-tumor activity, however, now it is recognized as a pleiotropic cytokine playing important roles in a host of biological processes and pathologies. To date, there are eight known members of the TNF-related cytokine family, TNF- $\alpha$ , TNF- $\beta$  (lymphotoxin- $\alpha$ ),

LT- $\beta$ , and ligands for the Fas receptor, CD30, CD27, CD40 and 4-1BB receptors. These proteins have conserved C-terminal sequences and variable N-terminal sequences which are often used as membrane anchors, with the exception of TNF- $\beta$ . Both TNF- $\alpha$  and TNF- $\beta$  function as homotrimers when they bind to TNF receptors.

TNF is produced by a number of cell types, including monocytes, fibroblasts, T cells, natural killer (NK) cells and predominately by activated macrophages. TNF- $\alpha$  has been reported to have a role in the rapid necrosis of tumors, immunostimulation, autoimmune disease, graft rejection, producing an anti-viral response, septic shock, cerebral malaria, cytotoxicity, protection against deleterious effects of ionizing radiation produced during a course of chemotherapy, such as denaturation of enzymes, lipid peroxidation and DNA damage (Nata et al, J. Immunol. 136(7):2483 (1987)), growth regulation, vascular endothelium effects, and metabolic effects. TNF- $\alpha$  also triggers endothelial cells to secrete various factors, including PAI-1, IL-1, GM-CSF and IL-6 to promote cell proliferation. In addition, TNF- $\alpha$  up-regulates various cell adhesion molecules such as E-Selectin, ICAM-1 and VCAM-1. TNF- $\alpha$  and the Fas ligand have also been shown to induce programmed cell death.

A related molecule, lymphotoxin (LT, also referred to as TNF- $\beta$ ), which is produced by activated lymphocytes shows a similar but not identical spectrum of biological activities as TNF. Two different types of LT have been found, LT- $\alpha$  and LT- $\beta$ . LT- $\alpha$  has many activities, including tumor necrosis, induction of an antiviral state, activation of polymorphonuclear leukocytes, induction of class I major histocompatibility complex antigens on endothelial cells, induction of adhesion molecules on endothelium and growth hormone stimulation (Ruddle, N. and Homer, R., Prog. Allergy, 40:162-182 (1988)).

The first step in the induction of the various cellular responses mediated by TNF or LT is their binding to specific cell surface or soluble receptors. Two distinct TNF receptors of approximately 55-KDa (TNF-R1) and 75-KDa (TNF-R2) have been identified (Hohman, H.P. et al., J. Biol. Chem., 264:14927-14934 (1989)), and human and mouse cDNAs corresponding to both receptor types have been isolated and characterized (Loetscher, H. et al., Cell, 61:351 (1990)). Both TNF-Rs share the typical structure of cell surface receptors including extracellular, transmembrane and intracellular regions.

These molecules exist not only in cell bound forms, but also in soluble forms, consisting of the cleaved extracellular domains of the intact receptors (Nophar et al., EMBO Journal, 9 (10):3269-76 (1990)). The extracellular domains of TNF-R1 and TNF-R2 share 28% identity and are characterized by four repeated cysteine-rich motifs with significant intersubunit sequence homology. The majority of cell types and tissues appear to express both TNF receptors and both receptors are active in signal transduction, however, they are able to mediate distinct cellular responses. Further, TNF-R2 was shown to exclusively mediate human T cell proliferation by TNF as shown in PCT WO 94/09137.

TNF-R1 dependent responses include accumulation of C-FOS, IL-6, and manganese superoxide dismutase mRNA, prostaglandin E2 synthesis, IL-2 receptor and MHC class I and II cell surface antigen expression, growth inhibition, and cytotoxicity. TNF-R1 also triggers second messenger systems such as phospholipase A<sub>2</sub>, protein kinase C, phosphatidylcholine-specific phospholipase C and sphingomyelinase (Pfefferk et al., Cell, 73:457-467 (1993)).

The receptor polypeptide of the present invention binds TNF, and in particular, TNF- $\beta$ . Further, the TNF receptor may also bind other ligands, including but not limited to Nerve Growth Factor, due to homology to a family of receptors and

antig ns which are involved in other critical biological processes. This family shows highly conserved cystein residues and includes the low affinity NGF r ceptor, which plays an important role in the regulation of growth and differentiation of nerve cells, the Fas receptor also called APO, a receptor which is involved in signalling for apoptosis and which, based on a study with mice deficient in its function, seems to play an important role in the etiology of a lupus-like disease, the TNF-R1, the B cell antigen CD40, and the T cell activation antigen CD27.

In accordance with one aspect of the present invention, there is provided a novel mature polypeptide which is a putative TNF receptor, as well as fragments, analogs and derivatives thereof. The polypeptide of the present invention is of human origin.

In accordance with another aspect of the present invention, there are provided isolated nucleic acid molecules encoding the polypeptide of the present invention, including mRNAs, DNAs, cDNAs, genomic DNA as well as antisense analogs thereof and biologically active and diagnostically or therapeutically useful fragments thereof.

In accordance with yet a further aspect of the present invention, there is provided a process for producing such polypeptides by recombinant techniques which comprises culturing recombinant prokaryotic and/or eukaryotic host cells, containing a nucleic acid sequence encoding a polypeptide of the present invention, under conditions promoting expression of said protein and subsequent recovery of said protein.

In accordance with yet a further aspect of the present invention, there is provided a process for utilizing such polypeptide, or polynucleotide encoding such polypeptide to screen for receptor antagonists and/or agonists and/or receptor ligands.

In accordance with yet a further aspect of the present invention, there are provided nucleic acid probes comprising nucleic acid molecules of sufficient length to specifically hybridize to the polypeptide of the present invention.

In accordance with still another aspect of the present invention, there is provided a process of using such agonists for treating conditions related to insufficient TNF receptor activity, for example, to inhibit tumor growth, to stimulate human cellular proliferation, e.g., T-cell proliferation, to regulate the immune response and antiviral responses, to protect against the effects of ionizing radiation, to protect against chlamidia infection, to regulate growth and to treat immunodeficiencies such as is found in HIV.

In accordance with another aspect of the present invention, there is provided a process of using such antagonists for treating conditions associated with over-expression of the TNF receptor, for example, for treating T-cell mediated autoimmune diseases such as AIDS, septic shock, cerebral malaria, graft rejection, cytotoxicity, cachexia, apoptosis and inflammation.

These and other aspects of the present invention should be apparent to those skilled in the art from the teachings herein.

The following drawings are illustrative of embodiments of the invention and are not meant to limit the scope of the invention as encompassed by the claims.

Figure 1 shows the cDNA sequence and corresponding deduced amino acid sequence of the polypeptide of the present invention. The initial 21 amino acids represent the putative leader sequence and are underlined. The standard one-letter abbreviations for amino acids are used. Sequencing was performed using a 373 automated DNA sequencer (Applied Biosystems, Inc.). Sequencing accuracy is predicted to be greater than 97% accurate.

Figure 2 illustrates an amino acid sequence alignment of the polypeptide of the present invention (upper line) and the human type 2 TNF receptor (lower line).

The term "gene" or "cistron" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons).

In accordance with an aspect of the present invention, there is provided an isolated nucleic acid (polynucleotide) which encodes for the mature polypeptide having the deduced amino acid sequence of Figure 1 (SEQ ID No. 2) or for the mature polypeptide encoded by the cDNA of the clone deposited as ATCC Deposit No. 75899 on September 28, 1994.

A polynucleotide encoding a polypeptide of the present invention may be obtained from human pulmonary tissue, hippocampus and adult heart. The polynucleotide of this invention was discovered in a cDNA library derived from human early passage fibroblasts (HSA 172 cells). It is structurally related to the human TNF-R2 receptor. It contains an open reading frame encoding a protein of 390 amino acid residues of which approximately the first 21 amino acids residues are the putative leader sequence such that the mature protein comprises 369 amino acids. The protein exhibits the highest degree of homology to a human type 2 TNF receptor with 39% identity and 46% similarity over an 88 amino acid stretch. Six conserved cysteines present in modules of 40 residues in all TNF receptors are conserved in this receptor.

The TNF receptor of the present invention is a soluble receptor and is secreted, however, it may also exist as a membrane bound receptor having a transmembrane region and an intra- and extracellular region. The polypeptide of the present invention may bind TNF and lymphotoxin ligands.



In accordance with an aspect of the present invention there is provided a polynucleotide which may be in the form of RNA or in the form of DNA, which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. The coding sequence which encodes the mature polypeptide may be identical to the coding sequence shown in Figure 1 (SEQ ID No. 1) or that of the deposited clone or may be a different coding sequence which coding sequence, as a result of the redundancy or degeneracy of the genetic code, encodes the same mature polypeptide as the DNA of Figure 1 (SEQ ID No. 1) or the deposited cDNA.

The polynucleotide which encodes for the mature polypeptide of Figure 1 (SEQ ID No. 2) or for the mature polypeptide encoded by the deposited cDNA may include: only the coding sequence for the mature polypeptide; the coding sequence for the mature polypeptide and additional coding sequence such as a leader or secretory sequence or a proprotein sequence; the coding sequence for the mature polypeptide (and optionally additional coding sequence) and non-coding sequence, such as introns or non-coding sequence 5' and/or 3' of the coding sequence for the mature polypeptide.

Thus, the term "polynucleotide encoding a polypeptide" encompasses a polynucleotide which includes only coding sequence for the polypeptide as well as a polynucleotide which includes additional coding and/or non-coding sequence.

The present invention further relates to variants of the hereinabove described polynucleotides which encode for fragments, analogs and derivatives of the polypeptide having the deduced amino acid sequence of Figure 1 (SEQ ID No. 2) or the polypeptide encoded by the cDNA of the deposited clone. The variant of the polynucleotide may be a naturally

occurring allelic variant of the polynucleotide or a non-naturally occurring variant of the polynucleotide.

Thus, the present invention includes polynucleotides encoding the same mature polypeptide as shown in Figure 1 (SEQ ID No. 2) or the same mature polypeptide encoded by the cDNA of the deposited clone as well as variants of such polynucleotides which variants encode for a fragment, derivative or analog of the polypeptide of Figure 1 (SEQ ID No. 2) or the polypeptide encoded by the cDNA of the deposited clone. Such nucleotide variants include deletion variants, substitution variants and addition or insertion variants.

As hereinabove indicated, the polynucleotide may have a coding sequence which is a naturally occurring allelic variant of the coding sequence shown in Figure 1 (SEQ ID No. 1) or of the coding sequence of the deposited clone. As known in the art, an allelic variant is an alternate form of a polynucleotide sequence which may have a substitution, deletion or addition of one or more nucleotides, which does not substantially alter the function of the encoded polypeptide.

The present invention also includes polynucleotides, wherein the coding sequence for the mature polypeptide may be fused in the same reading frame to a polynucleotide sequence which aids in expression and secretion of a polypeptide from a host cell, for example, a leader sequence which functions as a secretory sequence for controlling transport of a polypeptide from the cell. The polypeptide having a leader sequence is a preprotein and may have the leader sequence cleaved by the host cell to form the mature form of the polypeptide. The polynucleotides may also encode for a proprotein which is the mature protein plus additional 5' amino acid residues. A mature protein having a prosequence is a proprotein and is an inactive form of the protein. Once the prosequence is cleaved an active mature protein remains.

Thus, for example, the polynucleotide of the present invention may encode for a mature protein, or for a protein having a prosequence or for a protein having both a prosequence and a presequence (leader sequence).

The polynucleotides of the present invention may also have the coding sequence fused in frame to a marker sequence which allows for purification of the polypeptide of the present invention. The marker sequence may be a hexahistidine tag supplied by a pQE-9 vector to provide for purification of the mature polypeptide fused to the marker in the case of a bacterial host, or, for example, the marker sequence may be a hemagglutinin (HA) tag when a mammalian host, e.g. COS-7 cells, is used. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson, I., et al., Cell, 37:767 (1984)). The coding sequence may also be fused to a sequence which codes for a fusion protein such as an IgG Fc fusion protein.

The present invention further relates to polynucleotides which hybridize to the hereinabove-described sequences if there is at least 50% and preferably 70% identity between the sequences. The present invention particularly relates to polynucleotides which hybridize under stringent conditions to the hereinabove-described polynucleotides. As herein used, the term "stringent conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences. The polynucleotides which hybridize to the hereinabove described polynucleotides in a preferred embodiment encode polypeptides which retain substantially the same biological function or activity as the mature polypeptide encoded by the cDNA of Figure 1 (SEQ ID No. 1) or the deposited cDNA.

The deposit(s) referred to herein will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for purposes of

Pat nt Proc dure. These d posits are provided merely as convenience to those of skill in th art and are not an admission that a d posit is requir d under 35 U.S.C. §112. The sequence of the polynucleotid s contained in the deposited materials, as well as the amino acid sequence of the polypeptides encoded thereby, are incorporated herein by reference and are controlling in the event of any conflict with any description of sequences herein. A license may be required to make, use or sell the deposited materials, and no such license is hereby granted.

The present invention further relates to a polypeptide which has the deduced amino acid sequence of Figure 1 (SEQ ID No. 2) or which has the amino acid sequence encoded by the deposited cDNA, as well as fragments, analogs and derivatives of such polypeptide.

The terms "fragment," "derivative" and "analog" when referring to the polypeptide of Figure 1 (SEQ ID No. 2) or that encoded by the deposited cDNA, means a polypeptide which retains essentially the same biological function or activity as such polypeptide. Thus, an analog includes a proprotein which can be activated by cleavage of the proprotein portion to produce an active mature polypeptide.

The polypeptide of the present invention may be a recombinant polypeptide, a natural polypeptide or a synthetic polypeptide, preferably a recombinant polypeptide.

The fragment, derivative or analog of the polypeptide of Figure 1 (SEQ ID No. 2) or that encoded by the deposited cDNA may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the

half-life of the polypeptide (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature polypeptide, such as an IgG Fc fusion region peptide or leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

The polypeptides and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

The present invention also relates to vectors which include polynucleotides of the present invention, host cells which are genetically engineered with vectors of the invention and the production of polypeptides of the invention by recombinant techniques.

Host cells are genetically engineered (transduced or transformed or transfected) with the vectors of this invention which may be, for example, a cloning vector or an expression vector. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the nucleic

acid sequences of the present invention. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The polynucleotides of the present invention may be employed for producing polypeptides by recombinant techniques. Thus, for example, the polynucleotide may be included in any one of a variety of expression vectors for expressing a polypeptide. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences, e.g., derivatives of SV40; bacterial plasmids; phage DNA; baculovirus; yeast plasmids; vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other vector may be used as long as it is replicable and viable in the host.

The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art.

The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. As representative examples of such promoters, there may be mentioned: LTR or SV40 promoter, the *E. coli*. *lac* or *trp*, the phage lambda P<sub>L</sub> promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression.

In addition, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic

trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in E. coli.

The vector containing the appropriate DNA sequence as hereinabove described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the protein.

As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as E. coli, Streptomyces, Salmonella typhimurium; fungal cells, such as yeast; insect cells such as Drosophila S2 and Spodoptera Sf9; animal cells such as CHO, COS or Bowes melanoma; adenoviruses; plant cells, etc. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

More particularly, the present invention also includes recombinant constructs comprising one or more of the sequences as broadly described above. The constructs comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further comprises regulatory sequences, including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example. Bacterial: pQE70, pQE60, pQE-9 (Qiagen), pBS, pD10, phagescript, psiX174, pbluescript SK, pbsks, pNH8A, pNH16a, pNH18A, pNH46A (Stratagene); pTRC99a, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia). Eukaryotic: pWLNEO, pSV2CAT, pOG44, pXT1, pSG (Stratagene) pSVK3, pBPV, pMSG, pSVL (Pharmacia). However, any other plasmid or vector may be used as long as they are replicable and viable in the host.

Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. Two appropriate vectors are pKK232-8 and pCM7. Particular named bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda P<sub>R</sub>, P<sub>L</sub> and trp. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art.

In a further embodiment, the present invention relates to host cells containing the above-described constructs. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation (Davis, L., Digner, M., Battey, I., Basic Methods in Molecular Biology, (1986)).

The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively, the polypeptides of the invention can be synthetically produced by conventional peptide synthesizers.

Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, N.Y., (1989), the disclosure of which is hereby incorporated by reference.



Transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp that act on a promoter to increase its transcription. Examples including the SV40 enhancer on the late side of the replication origin bp 100 to 270, a cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, e.g., the ampicillin resistance gene of E. coli and S. cerevisiae TRP1 gene, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK),  $\alpha$ -factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product.

Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host. Suitable

prokaryotic hosts for transformation include E. coli, Bacillus subtilis, Salmonella typhimurium and various species within the genera Pseudomonas, Streptomyces, and Staphylococcus, although others may also be employed as a matter of choice.

As a representative but nonlimiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM1 (Promega Biotec, Madison, WI, USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is induced by appropriate means (e.g., temperature shift or chemical induction) and cells are cultured for an additional period.

Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification.

Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents, such methods are well known to those skilled in the art.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman, Cell, 23:175 (1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise

an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

The polypeptide of the present invention can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

The polypeptides of the present invention may be a naturally purified product, or a product of chemical synthetic procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect and mammalian cells in culture). Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. Polypeptides of the invention may also include an initial methionine amino acid residue.

The TNF receptor of the present invention was assayed for the ability to bind TNF- $\alpha$  and TNF- $\beta$ , however, the present invention also contemplates the ability of the receptor to bind other TNF-like proteins. Monoclonal antibodies specific to TNF- $\alpha$  and TNF- $\beta$  were prepared. These monoclonal antibodies were bound to TNF- $\alpha$  and TNF- $\beta$  and a control ELISA assay was performed to quantify the amount of monoclonal

antibody present. The TNF receptor was then bound to TNF- $\alpha$  and TNF- $\beta$  in the same way in which the monoclonal antibody was bound and another ELISA assay was performed. The TNF receptor was found to bind to TNF- $\beta$  just as strongly as the monoclonal antibody, while it only bound TNF- $\alpha$  two-thirds as strongly.

Fragments of the full length polynucleotide sequences of the present invention may be used as a hybridization probe for a cDNA library to isolate other genes which have a high sequence similarity to the polynucleotide sequence of the present invention or similar biological activity. Probes of this type generally have at least 50 bases, although they may have a greater number of bases. The probe may also be used as markers to identify a cDNA clone corresponding to a full length transcript and a genomic clone or clones that contain the complete polynucleotide sequence of the present invention including regulatory and promoter regions, exons, and introns. An example of a screen comprises isolating the coding region of the gene of the present invention by using the known DNA sequence to synthesize an oligonucleotide probe. Labeled oligonucleotides having a sequence complementary to that of the gene of the present invention are used to screen a library of human cDNA, genomic DNA or mRNA to determine which members of the library the probe hybridizes to.

This invention also provides a method of screening compounds to identify compounds which interact with the polypeptide of the present invention which comprises contacting a mammalian cell comprising an isolated DNA molecule encoding and expressing the polypeptide of the present invention with a plurality of compounds, determining those which activate or block the activation of the receptor, and thereby identifying compounds which specifically interact with, and activate or block the activation of the polypeptide of the present invention.

This invention also contemplates the use of the polynucleotide of the present invention as a diagnostic. For example, if a mutation is present, conditions would result from a lack of TNF receptor activity. Further, mutations which enhance TNF receptor activity would lead to diseases associated with an over-expression of the receptor, e.g., endotoxic shock. Mutated genes can be detected by comparing the sequence of the defective gene with that of a normal one. Subsequently one can verify that a mutant gene is associated with a disease condition or the susceptibility to a disease condition. That is, a mutant gene which leads to the underexpression of the TNF receptor would be associated with an inability of TNF to inhibit tumor growth.

Individuals carrying mutations in the polynucleotide of the present invention may be detected at the DNA level by a variety of techniques. Nucleic acids used for diagnosis may be obtained from a patient's cells which include, but are not limited to, blood, urine, saliva and tissue biopsy. The genomic DNA may be used directly for detection or may be amplified enzymatically by using PCR (Saiki et al., Nature, 324:163-166 (1986)) prior to analysis. RNA or cDNA may also be used for the same purpose. As an example, PCR primers complementary to the nucleic acid of the instant invention can be used to identify and analyze gene mutations. For example, deletions and insertions can be detected by a change in the size of the amplified product in comparison to the normal genotype. Point mutations can be identified by hybridizing amplified DNA to radiolabeled RNA or alternatively, radiolabeled TNF receptor antisense DNA sequences. Perfectly matched sequences can be distinguished from mismatched duplexes by RNase A digestion or by differences in melting temperatures. Such a diagnostic would be particularly useful for prenatal or even neonatal testing.

Sequence differences between the reference gene and "mutants" may be revealed by the direct DNA sequencing

method. In addition, cloned DNA segments may be used as probes to detect specific DNA segments. The sensitivity of this method is greatly enhanced when combined with PCR. For example, a sequencing primer used with double stranded PCR product or a single stranded template molecule generated by a modified PCR product. The sequence determination is performed by conventional procedures with radiolabeled nucleotides or by automatic sequencing procedures with fluorescent tags.

Sequence changes at the specific locations may be revealed by nuclease protection assays, such as RNase and S1 protection or the chemical cleavage method (for example, Cotton et al., PNAS, 85:4397-4401 (1985)).

The present invention further relates to a diagnostic assay which detects an altered level of a soluble form of the polypeptide of the present invention where an elevated level in a sample derived from a host is indicative of certain diseases. Assays available to detect levels of soluble receptors are well known to those of skill in the art, for example, radioimmunoassays, competitive-binding assays, Western blot analysis, and preferably an ELISA assay may be employed.

An ELISA assay initially comprises preparing an antibody specific to an antigen to the polypeptide of the present invention, preferably a monoclonal antibody. In addition a reporter antibody is prepared against the monoclonal antibody. To the reporter antibody is attached a detectable reagent such as radioactivity, fluorescence or in this example a horseradish peroxidase enzyme. A sample is now removed from a host and incubated on a solid support, e.g. a polystyrene dish, that binds the proteins in the sample. Any free protein binding sites on the dish are then covered by incubating with a non-specific protein such as bovine serum albumen. Next, the monoclonal antibody is incubated in the dish during which time the monoclonal antibodies attach to

any proteins of the present invention which are attached to the polystyrene dish. All unbound monoclonal antibody is washed out with buffer. The reporter antibody linked to horseradish peroxidase is now placed in the dish resulting in binding of the reporter antibody to any monoclonal antibody bound to the polypeptide of the present invention. Unattached reporter antibody is then washed out. Peroxidase substrates are then added to the dish and the amount of color developed in a given time period is a measurement of the amount of the protein of interest present in a given volume of patient sample when compared against a standard curve.

A competition assay may be employed wherein antibodies specific to the polypeptides of the present invention are attached to a solid support. Labeled TNF receptor polypeptides, and a sample derived from the host are passed over the solid support and the amount of label detected attached to the solid support can be correlated to a quantity in the sample. The soluble form of the receptor may also be employed to identify agonists and antagonists.

A thymocyte proliferation assay may be employed to identify both ligands and potential agonists and antagonists to the polypeptide of the present invention. For example, thymus cells are disaggregated from tissue and grown in culture medium. Incorporation of DNA precursors such as <sup>3</sup>H-thymidine or 5-bromo-2'-deoxyuridine (BrdU) is monitored as a parameter for DNA synthesis and cellular proliferation. Cells which have incorporated BrdU into DNA can be detected using a monoclonal antibody against BrdU and measured by an enzyme or fluorochrome-conjugated second antibody. The reaction is quantitated by fluorimetry or by spectrophotometry. Two control wells and an experimental well are set up. TNF- $\beta$  is added to all wells, while soluble receptors of the present invention are added to the experimental well. Also added to the experimental well is a compound to be screened. The ability of the compound to be

screened to inhibit the interaction of TNF- $\beta$  with the receptor polypeptides of the present invention may then be quantified. In the case of the agonists, the ability of the compound to enhance this interaction is quantified.

A determination may be made whether a ligand not known to be capable of binding to the polypeptide of the present invention can bind thereto comprising contacting a mammalian cell comprising an isolated molecule encoding a polypeptide of the present invention with a ligand under conditions permitting binding of ligands known to bind thereto, detecting the presence of any bound ligand, and thereby determining whether such ligands bind to a polypeptide of the present invention. Also, a soluble form of the receptor may be utilized in the above assay where it is secreted into the extra-cellular medium and contacted with ligands to determine which will bind to the soluble form of the receptor.

Other agonist and antagonist screening procedures involve, providing appropriate cells which express the receptor on the surface thereof. In particular, a polynucleotide encoding a polypeptide of the present invention is employed to transfect cells to thereby express the polypeptide. Such transfection may be accomplished by procedures as hereinabove described.

Thus, for example, such assay may be employed for screening for a receptor antagonist by contacting the cells which encode the polypeptide of the present invention with both the receptor ligand and a compound to be screened. Inhibition of the signal generated by the ligand indicates that a compound is a potential antagonist for the receptor, i.e., inhibits activation of the receptor.

The screening may be employed for determining an agonist by contacting such cells with compounds to be screened and determining whether such compounds generate a signal, i.e., activates the receptor.



Other screening techniques include the use of cells which express the polypeptide of the present invention (for example, transfected CHO cells) in a system which measures extracellular pH changes caused by receptor activation, for example, as described in Science, Volume 246, pages 181-296 (1989). In another example, potential agonists or antagonists may be contacted with a cell which expresses the polypeptide of the present invention and a second messenger response, e.g., signal transduction may be measured to determine whether the potential antagonist or agonist is effective.

Another screening technique involves expressing the receptor polypeptide wherein it is linked to phospholipase C or D. As representative examples of such cells, there may be mentioned endothelial cells, smooth muscle cells, embryonic kidney cells and the like. The screening for an antagonist or agonist may be accomplished as hereinabove described by detecting activation of the receptor or inhibition of activation of the receptor from the phospholipase second signal.

Antibodies may be utilized as both an agonist and antagonist depending on which part of the polypeptide of the present invention the antibody binds to. The antibody in one instance can bind to the active site and block ligand access. However, it has been observed that monoclonal antibodies directed against certain TNF receptors can act as specific agonists when binding to the extra-cellular domain of the receptor.

In addition to the antagonists identified above, oligonucleotides which bind to the TNF receptor may also act as TNF receptor antagonists. Alternatively, a potential TNF receptor antagonist may be a soluble form of the TNF receptor which contains the complete extra-cellular region of the TNF receptor and which binds to ligands to inhibit their biological activity.

Another potential TNF receptor antagonist is an antisense construct prepared using antisense technology. Antisense technology can be used to control gene expression through triple-helix formation or antisense DNA or RNA, both of which methods are based on binding of a polynucleotide to DNA or RNA. For example, the 5' coding portion of the polynucleotide sequence, which encodes for the mature polypeptides of the present invention, is used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription (triple helix -see Lee et al., Nucl. Acids Res., 6:3073 (1979); Cooney et al, Science, 241:456 (1988); and Dervan et al., Science, 251: 1360 (1991)), thereby preventing transcription and the production of TNF receptors. The antisense RNA oligonucleotide hybridizes to the mRNA *in vivo* and blocks translation of the mRNA molecule into the TNF receptor polypeptide (antisense - Okano, J. Neurochem., 56:560 (1991); Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988)). The oligonucleotides described above can also be delivered to cells such that the antisense RNA or DNA may be expressed *in vivo* to inhibit production of TNF receptors.

TNF receptor antagonists also include a small molecule which binds to and occupies the TNF receptor thereby making the receptor inaccessible to ligands which bind thereto such that normal biological activity is prevented. Examples of small molecules include but are not limited to small peptides or peptide-like molecules.

The TNF receptor agonists may be employed to stimulate ligand activities, such as inhibition of tumor growth and necrosis of certain transplantable tumors. The agonists may also be employed to stimulate cellular differentiation, for example, T-cell, fibroblasts and haemopoietic cell differentiation. Agonists to the TNF receptor may also

augment TNF's role in the host's defense against microorganisms and prevent related diseases (infections such as that from *L. monocytogenes*) and chlamidia. The agonists may also be employed to protect against the deleterious effects of ionizing radiation produced during a course of radiotherapy, such as denaturation of enzymes, lipid peroxidation, and DNA damage.

The agonists may also be employed to mediate an antiviral response, to regulate growth, to mediate the immune response and to treat immunodeficiencies related to diseases such as HIV.

Antagonists to the TNF receptor may be employed to treat autoimmune diseases, for example, graft versus host rejection and allograft rejection, and T-cell mediated autoimmune diseases such as AIDS. It has been shown that T-cell proliferation is stimulated via a type 2 TNF receptor. Accordingly, antagonizing the receptor may prevent the proliferation of T-cells and treat T-cell mediated autoimmune diseases.

The antagonists may also be employed to prevent apoptosis, which is the basis for diseases such as viral infection, rheumatoid arthritis, systemic lupus erythematosus, insulin-dependent diabetes mellitus, and graft rejection. Similarly, the antagonists may be employed to prevent cytotoxicity.

The antagonists to the TNF receptor may also be employed to treat B cell cancers which are stimulated by TNF.

Antagonists to the TNF receptor may also be employed to treat and/or prevent septic shock, which remains a critical clinical condition. Septic shock results from an exaggerated host response, mediated by protein factors such as TNF and IL-1, rather than from a pathogen directly. For example, lipopolysaccharides have been shown to elicit the release of TNF leading to a strong and transient increase of its serum concentration. TNF causes shock and tissue injury when

administered in excessive amounts. Accordingly, antagonists to the TNF receptor will block the actions of TNF and treat/prevent septic shock. These antagonists may also be employed to treat meningococemia in children which correlates with high serum levels of TNF.

Among other disorders which may be treated by the antagonists to TNF receptors, there are included, inflammation which is mediated by TNF receptor ligands, and the bacterial infections cachexia and cerebral malaria. The TNF receptor antagonists may also be employed to treat inflammation mediated by ligands to the receptor such as TNF.

The soluble TNF receptor and agonists and antagonists may be employed in combination with a suitable pharmaceutical carrier. Such compositions comprise a therapeutically effective amount of the soluble receptor or agonist or antagonist, and a pharmaceutically acceptable carrier or excipient. Such a carrier includes but is not limited to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The formulation should suit the mode of administration.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. In addition, the soluble form of the receptor and agonists and antagonists of the present invention may also be employed in conjunction with other therapeutic compounds.

The pharmaceutical compositions may be administered in a convenient manner such as by the oral, topical, intravenous, intraperitoneal, intramuscular, subcutaneous, intranasal or intradermal routes. The pharmaceutical

compositions are administered in an amount which is effective for treating and/or prophylaxis of the specific indication. In general, they are administered in an amount of at least about 10  $\mu\text{g/kg}$  body weight and in most cases they will be administered in an amount not in excess of about 8 mg/Kg body weight per day. In most cases, the dosage is from about 10  $\mu\text{g/kg}$  to about 1 mg/kg body weight daily, taking into account the routes of administration, symptoms, etc.

The TNF receptor and agonists and antagonists which are polypeptides may also be employed in accordance with the present invention by expression of such polypeptides *in vivo*, which is often referred to as "gene therapy."

Thus, for example, cells from a patient may be engineered with a polynucleotide (DNA or RNA) encoding a polypeptide *ex vivo*, with the engineered cells then being provided to a patient to be treated with the polypeptide. Such methods are well-known in the art. For example, cells may be engineered by procedures known in the art by use of a retroviral particle containing RNA encoding a polypeptide of the present invention.

Similarly, cells may be engineered *in vivo* for expression of a polypeptide *in vivo* by, for example, procedures known in the art. As known in the art, a producer cell for producing a retroviral particle containing RNA encoding the polypeptide of the present invention may be administered to a patient for engineering cells *in vivo* and expression of the polypeptide *in vivo*. These and other methods for administering a polypeptide of the present invention by such method should be apparent to those skilled in the art from the teachings of the present invention. For example, the expression vehicle for engineering cells may be other than a retrovirus, for example, an adenovirus which may be used to engineer cells *in vivo* after combination with a suitable delivery vehicle.

The sequences of the present invention are also valuable for chromosome identification. The sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. Moreover, there is a current need for identifying particular sites on the chromosome. Few chromosome marking reagents based on actual sequence data (repeat polymorphisms) are presently available for marking chromosomal location. The mapping of DNAs to chromosomes according to the present invention is an important first step in correlating those sequences with genes associated with disease.

Briefly, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp) from the cDNA. Computer analysis of the 3' untranslated region is used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids containing the human gene corresponding to the primer will yield an amplified fragment.

PCR mapping of somatic cell hybrids is a rapid procedure for assigning a particular DNA to a particular chromosome. Using the present invention with the same oligonucleotide primers, sublocalization can be achieved with panels of fragments from specific chromosomes or pools of large genomic clones in an analogous manner. Other mapping strategies that can similarly be used to map to its chromosome include *in situ* hybridization, prescreening with labeled flow-sorted chromosomes and preselection by hybridization to construct chromosome specific-cDNA libraries.

Fluorescence *in situ* hybridization (FISH) of a cDNA clone to a metaphase chromosomal spread can be used to provide a precise chromosomal location in one step. This technique can be used with cDNA as short as 500 or 600 bases; however, clones larger than 2,000 bp have a higher likelihood

of binding to a unique chromosomal location with sufficient signal intensity for simple detection. For example, 2,000 bp is good, 4,000 is better, and more than 4,000 is probably not necessary to get good results a reasonable percentage of the time. For a review of this technique, see Verma et al., Human Chromosomes: a Manual of Basic Techniques, Pergamon Press, New York (1988).

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. Such data are found, for example, in V. McKusick, Mendelian Inheritance in Man (available on line through Johns Hopkins University Welch Medical Library). The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes).

Next, it is necessary to determine the differences in the cDNA or genomic sequence between affected and unaffected individuals. If a mutation is observed in some or all of the affected individuals but not in any normal individuals, then the mutation is likely to be the causative agent of the disease.

With current resolution of physical mapping and genetic mapping techniques, a cDNA precisely localized to a chromosomal region associated with the disease could be one of between 50 and 500 potential causative genes. (This assumes 1 megabase mapping resolution and one gene per 20 kb).

The polypeptides, their fragments or other derivatives, or analogs thereof, or cells expressing them can be used as an immunogen to produce antibodies thereto. These antibodies can be, for example, polyclonal or monoclonal antibodies. The present invention also includes chimeric, single chain, and humanized antibodies, as well as Fab fragments, or the product of an Fab expression library. Various procedures

known in the art may be used for the production of such antibodies and fragments.

Antibodies generated against the polypeptides corresponding to a sequence of the present invention can be obtained by direct injection of the polypeptides into an animal or by administering the polypeptides to an animal, preferably a nonhuman. The antibody so obtained will then bind the polypeptides itself. In this manner, even a sequence encoding only a fragment of the polypeptides can be used to generate antibodies binding the whole native polypeptides. Such antibodies can then be used to isolate the polypeptide from tissue expressing that polypeptide.

For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler and Milstein, 1975, *Nature*, 256:495-497), the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, *Immunology Today* 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole, et al., 1985, in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96).

Techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce single chain antibodies to immunogenic polypeptide products of this invention. Also, transgenic mice may be used to express humanized antibodies to immunogenic polypeptide products of this invention.

The present invention will be further described with reference to the following examples; however, it is to be understood that the present invention is not limited to such examples. All parts or amounts, unless otherwise specified, are by weight.

In order to facilitate understanding of the following examples certain frequently occurring methods and/or terms will be described.



"Plasmids" are designated by a lower case p preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accord with published procedures. In addition, equivalent plasmids to those described are known in the art and will be apparent to the ordinarily skilled artisan.

"Digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements were used as would be known to the ordinarily skilled artisan. For analytical purposes, typically 1  $\mu$ g of plasmid or DNA fragment is used with about 2 units of enzyme in about 20  $\mu$ l of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50  $\mu$ g of DNA are digested with 20 to 250 units of enzyme in a larger volume. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37°C are ordinarily used, but may vary in accordance with the supplier's instructions. After digestion the reaction is electrophoresed directly on a polyacrylamide gel to isolate the desired fragment.

Size separation of the cleaved fragments is performed using 8 percent polyacrylamide gel described by Goeddel, D. et al., *Nucleic Acids Res.*, 8:4057 (1980).

"Oligonucleotides" refers to either a single stranded polydeoxynucleotide or two complementary polydeoxynucleotide strands which may be chemically synthesized. Such synthetic oligonucleotides have no 5' phosphate and thus will not ligate to another oligonucleotide without adding a phosphate with an ATP in the presence of a kinase. A synthetic

oligonucleotide will ligate to a fragment that has not been dephosphorylated.

"Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (Maniatis, T., et al., Id., p. 146). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units of T4 DNA ligase ("ligase") per 0.5 µg of approximately equimolar amounts of the DNA fragments to be ligated.

Unless otherwise stated, transformation was performed as described in the method of Graham, F. and Van der Eb, A., Virology, 52:456-457 (1973).

#### Example 1

##### Bacterial Expression and Purification of the TNF receptor

The DNA sequence encoding TNF receptor, ATCC # 75899, is initially amplified using PCR oligonucleotide primers corresponding to the 5' and 3' end sequences of the processed TNF receptor nucleic acid sequence (minus the signal peptide sequence). Additional nucleotides corresponding to TNF receptor gene are added to the 5' and 3' end sequences respectively. The 5' oligonucleotide primer has the sequence 5' GCCAGAG**GATCC**GAAACGTTTCCTCCAAAGTAC 3' (SEQ ID No. 3) contains a BamHI restriction enzyme site (bold) followed by 21 nucleotides of TNF receptor coding sequence starting from the presumed initiation codon. The 3' sequence 5' CGGCT**TCTAGA**ATTACCTATCATTTCTAAAAAT 3' (SEQ ID No. 4) contains complementary sequences to a Hind III site (bold) and is followed by 18 nucleotides of TNF receptor. The restriction enzyme sites correspond to the restriction enzyme sites on the bacterial expression vector pQE-9 (Qiagen, Inc. Chatsworth, CA). pQE-9 encodes antibiotic resistance (Amp'), a bacterial origin of replication (ori), an IPTG-regulatable promoter operator (P/O), a ribosome binding site (RBS), a 6-His tag and restriction enzyme sites. pQE-9 is then digested with BamHI and XbaI. The amplified sequences are ligated

into pQE-9 and are inserted in frame with the sequence encoding for the histidine tag and the RBS. The ligation mixture is then used to transform *E. coli* strain M15/rep 4 (Qiagen, Inc.) by the procedure described in Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, Cold Spring Laboratory Press, (1989). M15/rep4 contains multiple copies of the plasmid pREP4, which expresses the lacI repressor and also confers kanamycin resistance (Kan<sup>r</sup>). Transformants are identified by their ability to grow on LB plates and ampicillin/kanamycin resistant colonies are selected. Plasmid DNA is isolated and confirmed by restriction analysis. Clones containing the desired constructs are grown overnight (O/N) in liquid culture in LB media supplemented with both Amp (100 ug/ml) and Kan (25 ug/ml). The O/N culture is used to inoculate a large culture at a ratio of 1:100 to 1:250. The cells are grown to an optical density 600 (O.D.<sup>600</sup>) of between 0.4 and 0.6. IPTG ("Isopropyl-B-D-thiogalactopyranoside") is then added to a final concentration of 1 mM. IPTG induces by inactivating the lacI repressor, clearing the P/O leading to increased gene expression. Cells are grown an extra 3 to 4 hours. Cells are then harvested by centrifugation. The cell pellet is solubilized in the chaotropic agent 6 Molar Guanidine HCl. After clarification, solubilized TNF receptor is purified from this solution by chromatography on a Nickel-Chelate column under conditions that allow for tight binding by proteins containing the 6-His tag (Hochuli, E. et al., J. Chromatography 411:177-184 (1984)). TNF receptor (90% pure) is eluted from the column in 6 molar guanidine HCl pH 5.0 and for the purpose of renaturation adjusted to 3 molar guanidine HCl, 100mM sodium phosphate, 10 mMolar glutathione (reduced) and 2 mMolar glutathione (oxidized). After incubation in this solution for 12 hours the protein is dialyzed to 10 mMolar sodium phosphate.

Example 2Cloning and expression of TNF receptor and extracellular (soluble) TNF receptor using the baculovirus expression system

The DNA sequence encoding the full length TNF receptor protein, ATCC # 75899, was amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene. The 5' primer has the sequence 5' **GCGCGGATCCATGAACAAGTTGCTGTGCTGC** 3' (SEQ ID No. 5) and contains a BamHI restriction enzyme site (in bold) and which is just behind the first 21 nucleotides of the TNF receptor gene (the initiation codon for translation "ATG" is underlined).

The 3' primer has the sequence 5' GCGCTCTAGATTA CCTATCATTTCTAAAAATAAC 3' (SEQ ID No. 6) and 5' GCGCGGTACCTCAGTGGTTTGGGCTCCTCCC 3' (SEQ ID No. 7) and contains the cleavage site for the restriction endonuclease XbaI and 21 nucleotides complementary to the 3' non-translated sequence of the TNF receptor gene. The amplified sequences were isolated from a 1% agarose gel using a commercially available kit ("Geneclean", BIO 101 Inc., La Jolla, Ca.). The fragments were then digested with the endonucleases BamHI and XbaI and then purified again on a 1% agarose gel. This fragment is designated F2.

The vector pRG1 (modification of pVL941 vector, discussed below) was used for the expression of the TNF receptor proteins using the baculovirus expression system (for review see: Summers, M.D. and Smith, G.E. 1987, A manual of methods for baculovirus vectors and insect cell culture procedures, Texas Agricultural Experimental Station Bulletin No. 1555). This expression vector contains the strong polyhedrin promoter of the Autographa californica nuclear polyhedrosis virus (AcMNPV) followed by the recognition sites for the restriction endonucleases BamHI and XbaI. The polyadenylation site of the simian virus (SV)40 was used for

efficient polyadenylation. For an easy selection of recombinant viruses the beta-galactosidase gene from *E. coli* was inserted in the same orientation as the polyhedrin promoter followed by the polyadenylation signal of the polyhedrin gene. The polyhedrin sequences were flanked at both sides by viral sequences for the cell-mediated homologous recombination of cotransfected wild-type viral DNA. Many other baculovirus vectors could be used in place of pRG1 such as pAc373, pVL941 and pAcIM1 (Luckow, V.A. and Summers, M.D., *Virology*, 170:31-39).

The plasmid was digested with the restriction enzymes BamHI and XbaI. The DNA was then isolated from a 1% agarose gel using the commercially available kit ("Geneclean" BIO 101 Inc., La Jolla, Ca.). This vector DNA is designated V2.

Fragment F2 and the dephosphorylated plasmid V2 were ligated with T4 DNA ligase. *E. coli* HB101 cells were then transformed and cells identified that contained the plasmid (pBac TNF receptor) with the TNF receptor genes using the enzymes BamHI and XbaI. The sequence of the cloned fragment was confirmed by DNA sequencing.

5 µg of the plasmid pBac TNF receptor was cotransfected with 1.0 µg of a commercially available linearized baculovirus ("BaculoGold™ baculovirus DNA", Pharmingen, San Diego, CA.) using the lipofection method (Felgner et al. *Proc. Natl. Acad. Sci. USA*, 84:7413-7417 (1987)).

1 µg of BaculoGold™ virus DNA and 5 µg of the plasmid pBac TNF receptors were mixed in a sterile well of a microtiter plate containing 50 µl of serum free Grace's medium (Life Technologies Inc., Gaithersburg, MD). Afterwards 10 µl Lipofectin plus 90 µl Grace's medium were added, mixed and incubated for 15 minutes at room temperature. Then the transfection mixture was added dropwise to the Sf9 insect cells (ATCC CRL 1711) seeded in a 35 mm tissue culture plate with 1ml Grace' medium without serum. The plate was rocked back and forth to mix the newly

added solution. The plate was then incubated for 5 hours at 27°C. After 5 hours the transfection solution was removed from the plate and 1 ml of Grace's insect medium supplemented with 10% fetal calf serum was added. The plate was put back into an incubator and cultivation continued at 27°C for four days.

After four days the supernatant was collected and a plaque assay performed similar as described by Summers and Smith (supra). As a modification an agarose gel with "Blue Gal" (Life Technologies Inc., Gaithersburg) was used which allows an easy isolation of blue stained plaques. (A detailed description of a "plaque assay" can also be found in the user's guide for insect cell culture and baculovirology distributed by Life Technologies Inc., Gaithersburg, page 9-10).

Four days after the serial dilution, the viruses were added to the cells and blue stained plaques were picked with the tip of an Eppendorf pipette. The agar containing the recombinant viruses were then resuspended in an Eppendorf tube containing 200 µl of Grace's medium. The agar was removed by a brief centrifugation and the supernatant containing the recombinant baculoviruses was used to infect Sf9 cells seeded in 35 mm dishes. Four days later the supernatants of these culture dishes were harvested and then stored at 4°C.

Sf9 cells were grown in Grace's medium supplemented with 10% heat-inactivated FBS. The cells were infected with the recombinant baculovirus V-TNF receptor at a multiplicity of infection (MOI) of 2. Six hours later the medium was removed and replaced with SF900 II medium minus methionine and cysteine (Life Technologies Inc., Gaithersburg). 42 hours later 5 µCi of <sup>35</sup>S-methionine and 5 µCi <sup>35</sup>S cysteine (Amersham) were added. The cells are further incubated for 16 hours before they are harvested by centrifugation and the labelled proteins visualized by SDS-PAGE and autoradiography.

### Example 3

#### Expression of Recombinant TNF rec ptor in COS cells

The expression of plasmid, TNF rec ptor HA is derived from a vector pcDNAI/Amp (Invitrogen) containing: 1) SV40 origin of replication, 2) ampicillin resistance gene, 3) E.coli replication origin, 4) CMV promoter followed by a polylinker region, a SV40 intron and polyadenylation site. A DNA fragment encoding the entire TNF receptor precursor and a HA tag fused in frame to its 3' end is cloned into the polylinker region of the vector, therefore, the recombinant protein expression is directed under the CMV promoter. The HA tag correspond to an epitope derived from the influenza hemagglutinin protein as previously described (I. Wilson, H. Niman, R. Heighen, A Cherenson, M. Connolly, and R. Lerner, 1984, Cell 37, 767). The infusion of HA tag to the target protein allows easy detection of the recombinant protein with an antibody that recognizes the HA epitope.

The plasmid construction strategy is described as follows:

The DNA sequence encoding TNF receptor, ATCC # 75899, is constructed by PCR using two primers: the 5' primer 5' **GCCAGAGGATCCGCCACCATGAACAAGTTGCTGTGCTGC** 3' (SEQ ID No. 8) contains a BamHI site (bold) followed by 21 nucleotides of TNF receptor coding sequence starting from the initiation codon; the 3' sequence 5' **CGGCTTCTAGAATCAAGCGTAGTCTGGGACGTCGTATGGGTACCTATCATTCTAAAAAT** 3' (SEQ ID No. 9) contains complementary sequences to an XbaI site (bold), translation stop codon, HA tag and the last 18 nucleotides of the TNF receptor coding sequence (not including the stop codon). Therefore, the PCR product contains a BamHI site, TNF receptor coding sequence followed by HA tag fused in frame, a translation termination stop codon next to the HA tag, and an XbaI site. The PCR amplified DNA fragment and the vector, pcDNAI/Amp, are digested with BamHI and XbaI restriction enzymes and ligated. The ligation mixture is transformed into

*E. coli* strain SURE (Stratagen Cloning Systems, La Jolla, CA) the transformed culture is plated on ampicillin media plates and resistant colonies are selected. Plasmid DNA is isolated from transformants and examined by restriction analysis for the presence of the correct fragment. For expression of the recombinant TNF receptor, COS cells are transfected with the expression vector by DEAE-DEXTRAN method (J. Sambrook, E. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Laboratory Press, (1989)). The expression of the TNF receptor HA protein is detected by radiolabelling and immunoprecipitation method (E. Harlow, D. Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, (1988)). Cells are labelled for 8 hours with <sup>35</sup>S-cysteine two days post transfection. Culture media are then collected and cells are lysed with detergent (RIPA buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 1% NP-40, 0.5% DOC, 50mM Tris, pH 7.5) (Wilson, I. et al., Id. 37:767 (1984))). Both cell lysate and culture media are precipitated with a HA specific monoclonal antibody. Proteins precipitated are analyzed on 15% SDS-PAGE gels.

Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, within the scope of the appended claims, the invention may be practiced otherwise than as particularly described.



## SEQUENCE LISTING

- (1) GENERAL INFORMATION:
- (i) APPLICANT: GREENE, ET AL.
- (ii) TITLE OF INVENTION: Human Tumor Necrosis Factor  
Receptor
- (iii) NUMBER OF SEQUENCES: 9
- (iv) CORRESPONDENCE ADDRESS:
- (A) ADDRESSEE: CARELLA, BYRNE, BAIN, GILFILLAN,  
CECCHI, STEWART & OLSTEIN
- (B) STREET: 6 BECKER FARM ROAD
- (C) CITY: ROSELAND
- (D) STATE: NEW JERSEY
- (E) COUNTRY: USA
- (F) ZIP: 07068
- (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: 3.5 INCH DISKETTE
- (B) COMPUTER: IBM PS/2
- (C) OPERATING SYSTEM: MS-DOS
- (D) SOFTWARE: WORD PERFECT 5.1
- (vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER:
- (B) FILING DATE: Concurrently
- (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA
- (A) APPLICATION NUMBER:
- (B) FILING DATE:

- (viii) ATTORNEY/AGENT INFORMATION:
- (A) NAME: FERRARO, GREGORY D.
  - (B) REGISTRATION NUMBER: 36,134
  - (C) REFERENCE/DOCKET NUMBER: 325800-266
- (ix) TELECOMMUNICATION INFORMATION:
- (A) TELEPHONE: 201-994-1700
  - (B) TELEFAX: 201-994-1744
- (2) INFORMATION FOR SEQ ID NO:1:
- (i) SEQUENCE CHARACTERISTICS
- (A) LENGTH: 1173 BASE PAIRS
  - (B) TYPE: NUCLEIC ACID
  - (C) STRANDEDNESS: SINGLE
  - (D) TOPOLOGY: LINEAR
- (ii) MOLECULE TYPE: cDNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```

ATGAACAAGT TGCTGTGCTG CGCGCTCGTG TTTCTGGACA TCTCCATTAA GTGGACCACC      60
CAGGAAACGT TTCCTCCAAA GTACCTTCAT TATGACGAAG AAACCTCTCA TCAGCTGTTG     120
TGTGACAAAT GTCCTCCTGG TACCTACCTA AAACAACACT GTACAGCAAA GTGGAAGACC     180
GTGTGCGCCC CTTGCCCTGA CCACTACTAC ACAGACAGCT GGCACACCAG TGACGAGTGT     240
CTATACTGCA GCCCCGTGTG CAAGGAGCTG CAGTACGTCA AGCAGGAGTG CAATCGCACC     300
CACAACCGCG TGTGCGAATG CAAGGAAGGG CGCTACCTTG AGATAGAGTT CTGCTTGAAA     360
CATAGGAGCT GCCCTCCTGG ATTTGGAGTG GTGCAAGCTG GAACCCAGAG GCGAAATACA     420
GTTTGCAAAA GATGTCCAGA TGGGTTCTTC TCAAATGAGA CGTCATCTAA AGCACCCCTGT     480
AGAAAACACA CAAATTGCAG TGTCTTTGGT CTCCTGCTAA CTCAGAAAGG AAATGCAACA     540
CACGACAACA TATGTTCCGG AAACAGTGAA TCAACTCAAA AATGTGGAAT AGATGTTACC     600
CTGTGTGAGG AGGCATTCTT CAGGTTTGCT GTTCCTACAA AGTTTACGCC TAACTGGCTT     660
AGTGTCTTGG TAGACAATTT GCCTGGCACC AAAGTAAACG CAGAGAGTGT AGAGAGGATA     720
AAACGGCAAC ACAGCTCACA AGAACAGACT TTCCAGCTGC TGAAGTTATG GAAACATCAA     780
AACAAAGACC AAGATATAGT CAAGAAGATC ATCCAAGATA TTGACCTCTG TGAAAACAGC     840
GTGCAGCGGC ACATTGGACA TGCTAACCTC ACCTTCGAGC AGCTTCGTAG CTTGATGGAA     900
AGCTTACCGG GAAAGAAAGT GGGAGCAGAA GACATTGAAA AAACAATAAA GGCATGCAAA     960
CCCAGTGACC AGATCCTGAA GCTGCTCACT TTGTGGCGAA TAAAAAATGG CGACCAAGAC    1020

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ACCTTGAAGG GCCTAATGCA CGCACTAAAG CACTCAAAGA CGTACCACTT TCCCACAAAC 1080  
 TGTCACTCAG AGTCTAAAGA AGACCATCAG GTTCCTTCAC AGCTTCACAA TGTACAAATT 1140  
 GTATCAGAAG TTATTTT TAG AAATGATAGG TAA 1173

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 390 AMINO ACIDS  
 (B) TYPE: AMINO ACID  
 (C) STRANDEDNESS:  
 (D) TOPOLOGY: LINEAR

## (ii) MOLECULE TYPE: PROTEIN

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met	Asn	Lys	Leu	Leu	Cys	Cys	Ala	Leu	Val	Phe	Leu	Asp	Ile	Ser	-20	-15	-10
Ile	Lys	Trp	Thr	Thr	Gln	Glu	Thr	Phe	Pro	Pro	Lys	Tyr	Leu	His	-5	1	5
Tyr	Asp	Glu	Glu	Thr	Ser	His	Gln	Leu	Leu	Cys	Asp	Lys	Cys	Pro	10	15	20
Pro	Gly	Thr	Tyr	Leu	Lys	Gln	His	Cys	Thr	Ala	Lys	Trp	Lys	Thr	25	30	35
Val	Cys	Ala	Pro	Cys	Pro	Asp	His	Tyr	Tyr	Thr	Asp	Ser	Trp	His	40	45	50
Thr	Ser	Asp	Glu	Cys	Leu	Tyr	Cys	Ser	Pro	Val	Cys	Lys	Glu	Leu	55	60	65
Gln	Tyr	Val	Lys	Gln	Glu	Cys	Asn	Arg	Thr	His	Asn	Arg	Val	Cys	70	75	80
Glu	Cys	Lys	Glu	Gly	Arg	Tyr	Leu	Glu	Ile	Glu	Phe	Cys	Leu	Lys	85	90	95
His	Arg	Ser	Cys	Pro	Pro	Gly	Phe	Gly	Val	Val	Gln	Ala	Gly	Thr	100	105	110
Pro	Glu	Arg	Asn	Thr	Val	Cys	Lys	Arg	Cys	Pro	Asp	Gly	Phe	Phe	115	120	125

Ser Asn Glu Thr Ser Ser Lys Ala Pro Cys Arg Lys His Thr Asn		
130	135	140
Cys S r Val Phe Gly Leu Leu Leu Thr Gln Lys Gly Asn Ala Thr		
145	150	155
His Asp Asn Ile Cys Ser Gly Asn Ser Glu Ser Thr Gln Lys Cys		
160	165	170
Gly Ile Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg Phe Ala		
175	180	185
Val Pro Thr Lys Phe Thr Pro Asn Trp Leu Ser Val Leu Val Asp		
190	195	200
Asn Leu Pro Gly Thr Lys Val Asn Ala Glu Ser Val Glu Arg Ile		
205	210	215
Lys Arg Gln His Ser Ser Gln Glu Gln Thr Phe Gln Leu Leu Lys		
220	225	230
Leu Trp Lys His Gln Asn Lys Asp Gln Asp Ile Val Lys Lys Ile		
235	240	245
Ile Gln Asp Ile Asp Leu Cys Glu Asn Ser Val Gln Arg His Ile		
250	255	260
Gly His Ala Asn Leu Thr Phe Glu Gln Leu Arg Ser Leu Met Glu		
265	270	275
Ser Leu Pro Gly Lys Lys Val Gly Ala Glu Asp Ile Glu Lys Thr		
280	285	290
Ile Lys Ala Cys Lys Pro Ser Asp Gln Ile Leu Lys Leu Leu Ser		
295	300	305
Leu Trp Arg Ile Lys Asn Gly Asp Gln Asp Thr Leu Lys Gly Leu		
310	315	320
Met His Ala Leu Lys His Ser Lys Thr Tyr His Phe Pro Thr Asn		
325	330	335
Cys His Ser Glu Ser Lys Glu Asp His Gln Val Pro Ser Gln Leu		
340	345	350
His Asn Val Gln Ile Val Ser Glu Val Ile Phe Arg Asn Asp Arg		
355	360	365

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS
  - (A) LENGTH: 33 BASE PAIRS
  - (B) TYPE: NUCLEIC ACID
  - (C) STRANDEDNESS: SINGLE
  - (D) TOPOLOGY: LINEAR
- (ii) MOLECULE TYPE: Oligonucleotide
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GCCAGAGGAT CCGAAACGTT TCCTCCAAAG TAC

33

- (2) INFORMATION FOR SEQ ID NO:4:
  - (i) SEQUENCE CHARACTERISTICS
    - (A) LENGTH: 33 BASE PAIRS
    - (B) TYPE: NUCLEIC ACID
    - (C) STRANDEDNESS: SINGLE
    - (D) TOPOLOGY: LINEAR
  - (ii) MOLECULE TYPE: Oligonucleotide
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CGGCTTCTAG AATTACCTAT CATTTCTAAA AAT

33

- (2) INFORMATION FOR SEQ ID NO:5:
  - (i) SEQUENCE CHARACTERISTICS
    - (A) LENGTH: 31 BASE PAIRS
    - (B) TYPE: NUCLEIC ACID
    - (C) STRANDEDNESS: SINGLE
    - (D) TOPOLOGY: LINEAR
  - (ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCGCGGATCC ATGAACAAGT TGCTGTGCTG C 31

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 34 BASE PAIRS

(B) TYPE: NUCLEIC ACID

(C) STRANDEDNESS: SINGLE

(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCGCTCTAGA TTACCTATCA TTTCTAAAAA TAAC 34

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 31 BASE PAIRS

(B) TYPE: NUCLEIC ACID

(C) STRANDEDNESS: SINGLE

(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCGCGGTACC TCAGTGTTT GGGCTCCTCC C 31

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 39 BASE PAIRS
- (B) TYPE: NUCLEIC ACID
- (C) STRANDEDNESS: SINGLE
- (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCCAGAGGAT CCGCCACCAT GAACAAGTTG CTGTGCTGC

39

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 60 BASE PAIRS
- (B) TYPE: NUCLEIC ACID
- (C) STRANDEDNESS: SINGLE
- (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CGGCTTCTAG AATCAAGCGT AGTCTGGGAC GTCGTATGGG TACCTATCAT TTCTAAAAAT

60

WHAT IS CLAIMED IS:

1. An isolated polynucleotide selected from the group consisting of:
  - (a) a polynucleotide encoding the polypeptide having the deduced amino acid sequence of SEQ ID No. 2 or a fragment, analog or derivative of said polypeptide;
  - (b) a polynucleotide encoding the polypeptide having the amino acid sequence encoded by the cDNA contained in ATCC Deposit No. 75899 or a fragment, analog or derivative of said polypeptide.
2. The polynucleotide of Claim 1 wherein the polynucleotide is DNA.
3. The polynucleotide of Claim 1 wherein the polynucleotide is RNA.
4. The polynucleotide of Claim 1 wherein the polynucleotide is genomic DNA.
5. The polynucleotide of Claim 2 wherein said polynucleotide encodes the polypeptide having the deduced amino acid sequence of SEQ ID No. 2.
6. The polynucleotide of Claim 2 wherein said polynucleotide encodes the polypeptide encoded by the cDNA of ATCC Deposit No. 75899.
7. The polynucleotide of Claim 1 having the coding sequence of the polypeptide shown in SEQ ID No. 2.
8. The polynucleotide of Claim 2 having the coding sequence of the polypeptide deposited as ATCC Deposit No. 75899.
9. A vector containing the DNA of Claim 2.
10. A host cell genetically engineered with the vector of Claim 9.
11. A process for producing a polypeptide comprising: expressing from the host cell of Claim 10 the polypeptide encoded by said DNA.



12. A process for producing cells capable of expressing a polypeptide comprising genetically engineering cells with the vector of Claim 9.
13. An isolated DNA hybridizable to the DNA of Claim 2 and encoding a polypeptide having TNF receptor activity.
14. A polypeptide selected from the group consisting of (i) a polypeptide having the deduced amino acid sequence of SEQ ID No. 2 and fragments, analogs and derivatives thereof and (ii) a polypeptide encoded by the cDNA of ATCC Deposit No. 75899 and fragments, analogs and derivatives of said polypeptide.
15. The polypeptide of Claim 14 wherein the polypeptide has the deduced amino acid sequence of SEQ ID No. 2.
16. An antibody against the polypeptide of claim 14.
17. A compound which activates the polypeptide of claim 14.
18. A compound which activates the polypeptide of claim 14.
19. A method for the treatment of a patient having need to activate a TNF receptor comprising: administering to the patient a therapeutically effective amount of the compound of claim 18.
20. A method for the treatment of a patient having need to inhibit a TNF receptor comprising: administering to the patient a therapeutically effective amount of the compound of claim 17.
21. The method of claim 19 wherein said therapeutically effective amount of the compound is administered by providing to the patient DNA encoding said compound and expressing said compound *in vivo*.
22. The method of claim 20 wherein said therapeutically effective amount of the compound is administered by providing to the patient DNA encoding said compound and expressing said compound *in vivo*.

23. A method for identifying agonists and antagonists to the polypeptide of claim 14 comprising:

(a) combining TNF-receptor, a reaction mixture containing cells which proliferate in response to a ligand known to bind to the TNF-receptor and a compound to be screened under conditions where the ligand binds to the TNF receptor polypeptide; and

(b) determining whether the compound inhibits or enhances the interaction of the TNF receptor and the ligand.

24. A process for diagnosing a disease or a susceptibility to a disease related to an under-expression of the polypeptide of claim 14 comprising:

determining a mutation in the nucleic acid sequence encoding said polypeptide.

25. The polypeptide of Claim 14 wherein the polypeptide is a soluble fragment of the TNF receptor and is capable of binding a ligand for the receptor.

26. A diagnostic process comprising:

analyzing for the presence of the polypeptide of claim 25 in a sample derived from a host.

27. A process for determining whether a ligand not known to be capable of binding to the polypeptide of claim 14 can bind thereto comprising:

contacting a mammalian cell which expresses the receptor with a potential ligand;

detecting the presence of the ligand which binds to the receptor; and

determining whether the ligand binds to the receptor.

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FIG. 1A

1 ATGAACAAGTTGCTGTGCTGCGCGCTCGTGTTCCTGGACAT  
M N K L L C C A L V F L D I  
42 CTCCATTAAAGTGGACCACCCAGGAAACGTTTCCTCCAAAGT  
S I K W T T Q E T F P P K  
83 ACCTTCATTATGACGAAGAAACCTCTCATCAGCTGTTGTGT  
Y L H Y D E E T S H Q L L C  
124 GACAAATGTCTCTCTGGTACCTACCTAAAACAACACTGTAC  
D K C P P G T Y L K Q H C T  
165 AGCAAAGTGGAAGACCGTGTGCGCCCCCTTGCCCTGACCACT  
A K W K T V C A P C P D H  
206 ACTACACAGACAGCTGGCACACCAGTGACGAGTGTCTATAC  
Y Y T D S W H T S D E C L Y  
247 TGCAGCCCCGTGTGCAAGGAGCTGCAGTACGTCAAGCAGGA  
C S P V C K E L Q Y V K Q E  
288 GTGCAATCGCACCCACAACCGCGTGTGCGAATGCAAGGAAG  
C N R T H N R V C E C K E  
329 GCGCTACCTTGAGATAGAGTTCTGCTTGAAACATAGGAGC  
G R Y L E I E F C L K H R S  
370 TGCCCTCCTGGATTTGGAGTGGTGCAAGCTGGAACCCCAGA  
C P P G F G V V Q A G T P E  
411 GCGAAATACAGTTTGCAAAAGATGTCCAGATGGGTTCCTCT  
R N T V C K R C P D G F F  
452 CAAATGAGACGTCATCTAAAGCACCCCTGTAGAAAACACACA  
S N E T S S K A P C R K H T  
493 AATTGCAGTGTCTTTGGTCTCCTGCTAACTCAGAAAGGAAA  
N C S V F G L L L T Q K G N  
534 TGCAACACACGACAACATATGTTCCGGAACAGTGAATCAA  
A T H D N I C S G N S E S

MATCH WITH FIG. 1B

SUBSTITUTE SHEET (RULE 26)

2 / 5 MATCH WITH FIG. 1A

575 CTCAAAAATGTGGAATAGATGTTACCCCTGTGTGAGGAGGCA  
T Q K C G I D V T L C E E A  
616 TTCTTCAGGTTTGCTGTTCCTACAAAGTTTACGCCTAACTG  
F F R F A V P T K F T P N W  
657 GCTTAGTGICTTGGTAGACAATTTGCCTGGCACCAAAGTAA  
L S V L V D N L P G T K V  
698 ACGCAGAGAGTGTAGAGAGGATAAAACGCCAACACAGCTCA  
N A E S V E R I K R Q H S S  
739 CAAGAACAGACTTTCAGCTGCTGAAGTTATGGAAACATCA  
Q E Q T F Q L L K L W K H Q  
780 AAACAAAGACCAAGATATAGTCAAGAAGATCATCCAAGATA  
N K D Q D I V K K I I Q D  
821 TTGACCTCTGTGAAAACAGCGTGCAGCGGCACATTGGACAT  
I D L C E N S V Q R H I G H  
862 GCTAACCTCACCTTCGAGCAGCTTCGTAGCTTGATGGAAAG  
A N L T F E Q L R S L M E S  
903 CTTACCGGGAAAGAAAGTGGGAGCAGAAGACATTGAAAAAA  
L P G K K V G A E D I E K  
944 CAATAAAGGCATGCAAACCCAGTGACCAGATCCTGAAGCTG  
T I K A C K P S D Q I L K L  
985 CTCAGTTTGTGGCGAATAAAAAATGGCGACCAAGACACCTT  
L S L W R I K N G D Q D T L  
1026 GAAGGGCCTAATGCACGCACTAAAGCACTCAAAGACGTACC  
K G L M H A L K H S K T Y  
1067 ACTTTCCCAAACTGTCACTCAGAGTCTAAAGAAGACCAT  
H F P T N C H S E S K E D H  
1108 CAGGTTCCTTCACAGCTTCACAATGTACAAATTGTATCAGA  
Q V P S Q L H N V Q I V S E  
1149 AGTTATTTTITAGAAATGATAGGTAA  
V I F R N D R .

FIG. 1B

## FIG. 2A

tnfr2.msf(TNFR2_LIKE)	1	.....MNKLL	CCALVFLDIS	IKWTTQETFP	P.....	KYLHYDEETS	50
tnfr2.msf(TNR2_HUMAN)		MAPVAVWAAL	AVGLELWAAA	HALPAQVAF	PYAPEPGSTC	RLREYYDQTA	
Consensus		-----L	---L-----	-----Q--F-	P-----	-----Y---T-	
tnfr2.msf(TNFR2_LIKE)	51	HQLLCDKCPP	GTYLKQHCTA	KWKTVCAPCP	DHYTDSWHT	SDECLYCSVP	100
tnfr2.msf(TNR2_HUMAN)		.QMCCSKCSP	GQHAKVFCTK	TSDTVCDSC	DSTYTQLWNW	VPECLSCGSR	
Consensus		-Q--C-KC-P	G---K--CT-	---TVC--C-	D--YT--W--	--ECL-C---	
tnfr2.msf(TNFR2_LIKE)	101	CKELQYVKQE	CNRTHNRVCE	CKEGRYLEIE	.....FCLK	HRSCPPGFGV	150
tnfr2.msf(TNR2_HUMAN)		CSSDQVETQA	CTREQNRICT	CRPGWYCAL	KQEGCRLCAP	LRKCRPGFGV	
Consensus		C---Q---Q-	C-R--NR-C-	C--G-Y----	-----C--	-R-C-PGFGV	
tnfr2.msf(TNFR2_LIKE)	151	VOAGTPERNT	VCKRCPDGFF	SNETSSKAPC	RKHTNCSVFG	LLLTQKGNAT	200
tnfr2.msf(TNR2_HUMAN)		ARPGTETSDV	VCKPCAPGTF	SNTTSSSTDIC	RPHQICNVVA	I....PGNAS	
Consensus		---GT-----	VCK-C--G-F	SN-TSS---C	R-H--C-V--	-----GNA-	
tnfr2.msf(TNFR2_LIKE)	201	HDNIC.....	.....	.....SGNSE	STQKCGIDVT	LCEEAF...	250
tnfr2.msf(TNR2_HUMAN)		RDAVCTSTSP	TRSMAPGAVH	LPQPVSTRSQ	HTQPTPEPST	APSTSFLLPM	
Consensus		-D--C-----	-----	-----S--S-	-TQ-----T	-----F----	

MATCH WITH FIG. 2B

## FIG. 2B

MATCH WITH FIG. 2A

```

tnfr2.msf{TNFR2_LIKE}          251          300
tnfr2.msf{TNR2_HUMAN}          .RFAVPTKFT PNWLSVLVDN LPGTKVNAES VERIKR....
Consensus                      --FA-P----- L-----VN--- ----K-----

tnfr2.msf{TNFR2_LIKE}          301          350
tnfr2.msf{TNR2_HUMAN}          .QHSSQEQTFF QLLKLWKHQN KDQDIV.... .KKIIQDIDL CENSVQRHIG
Consensus                      --K-----Q- --Q----- ----R-----

tnfr2.msf{TNFR2_LIKE}          351          400
tnfr2.msf{TNR2_HUMAN}          HANLTFEQLR SLMESLPGKK VGAEDIEKTI KACKPSDQIL KLLSLWRIKN
Consensus                      --N-----Q-- ---S--G-- ----R-----

tnfr2.msf{TNFR2_LIKE}          401          450
tnfr2.msf{TNR2_HUMAN}          GDQDTLKGLM HALKHSKTYH FPTNCHSESK EDHQVPSQLH NVQIVSEVIF
Consensus                      -----S-T-- -A---S-T-- -----SES- -D-QVP-----S-----

tnfr2.msf{TNFR2_LIKE}          451          475
tnfr2.msf{TNR2_HUMAN}          RNDR..... PETLLGSTEE KPLPLGVPDA GMKPS
Consensus                      -----S-T-- -A---S-T-- -----SES- -D-QVP-----S-----

```

MATCH WITH FIG. 2C

MATCH WITH FIG. 2B

FIG. 2C

Query: 38 QLLCDKCPPGTYLKQHCTAKWKTVCAPCPDHYYTDSWHTSDECLYCSPVCKELQVVKQEC 97  
 Q+ C KC PG + K CT TVC C D YT W+ ECL C C Q Q C

Sbjct: 29 QMCCSKCSPGQHAKVFC TKTSDTVCDSCEDSTYTQLWNWVPECLSCGSRCSDDQVETQAC 88

Query: 98 NRTHNRVCECKEGRYLEIEFCLKHRSCPP 126  
 R NR+C C+ G Y + R C P

Sbjct: 89 TREQNRICTCRPGWYCALSKQEGCRLCAP 117

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Query: 118 CLKHRSCPPGFGVVQAGTPERNTVCKRCPDGFSSNETSSKAPCRKHTNCSVFGL 171  
 C R C PGFGV + GT + VCK C G FSN TSS CR H C+V +

Sbjct: 115 CAPLRKCRPGFGVARPGTETSDVWCKPCAPGTFSTSTSDICRPHQICNVVAI 168

Query: 177 GNATHDNICSGNS 189  
 GNA+ D +C+ S

Sbjct: 170 GNASMDAVCTSTS 182

Query: 363 SESKEDHQVP 372  
 SES +D QVP

Sbjct: 391 SESPKEQVP 400

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/03216

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : Please See Extra Sheet.

US CL : 536/23.1, 24.31; 530/351; 435/69.5, 240.1, 320.1

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1, 24.31; 530/351; 435/69.5, 240.1, 320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog, Biosis

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DNA and Cell Biology, Vol. 9, No. 10, issued 1990, A. Himmler et al., "Molecular cloning and expression of human and rat tumor necrosis factor receptor chain (p60) and its soluble derivative, tumor necrosis factor-binding protein", pages 705-715, see entire document and especially Fig. 3.	1-15, 25
A	J. Immunol., Vol. 147, No. 9, issued 01 November 1991, D. Camerini et al., "The T cell activation antigen CD27 is a member of the nerve growth factor/tumor necrosis factor receptor gene family" pages 3165-3169, see especially Fig. 6.	1-15, 25

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	* T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* A* document defining the general state of the art which is not considered to be of particular relevance	* X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
* E* earlier document published on or after the international filing date	* Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
* L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* &*	document member of the same patent family
* O* document referring to an oral disclosure, use, exhibition or other means		
* P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

15 JUNE 1995

Date of mailing of the international search report

30 JUN 1995

Name and mailing address of the ISA/US  
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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/03216

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Cell, Vol. 68, issued 07 February 1992, H. Durkop et al., "Molecular cloning and expression of a new member of the nerve growth factor receptor family that is characteristic for Hodgkin's Disease", pages 421-427, see especially Fig. 3.	1-15, 25
A	Eur. J. Biochem., Vol. 220, issued April 1994, X. Van Ostade et al., "Human tumor necrosis factor mutants with preferential binding to and activity on either the R55 or R75 receptor", pages 771-779, see entire document.	1-15, 25

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/03216

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-15, 25

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/03216

## A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

C12N 15/00, 5/10, 5/00, 15/11, 15/28, 15/09, 15/63; C07K 14/00, 14/525; C12P 21/06

## BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-15 and 25, drawn to polynucleotides encoding TNF receptor, TNF receptor, vectors, hosts, methods of producing TNF receptor.

Group II, claim 16, drawn to antibodies against TNF receptor.

Group III, claims 17 and 18, drawn to a compound that activates TNF receptor.

Group IV, claims 19 and 20, drawn to a method of treatment of a patient in need of TNF receptor activation or inhibition by administering the compound of Group III.

Group V, claims 21 and 22, drawn to a method of treatment of a patient in need of TNF receptor activation or inhibition by administering the DNA encoding the compound of Group III.

Group VI, claim 23, drawn to a method of identifying agonists and antagonists of TNF receptor.

Group VII, claim 24, drawn to a process for diagnosing a disease related to underexpression of the TNF receptor via the detection of a mutation in the nucleic acid encoding the TNF receptor.

Group VIII, claim 26, drawn to a diagnostic process comprising analyzing the presence of soluble TNF receptor.

Group IX, claim 27, drawn to a process for determining unknown ligands which bind to TNF receptor.

The inventions listed as Groups I-IX do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The polynucleotides encoding TNF receptor and the TNF receptor of Group I, the antibody of Group II, and the compound of Group III are distinct because these compounds differ in composition and function. The nucleic acid or TNF receptor can be used in any of the methods of Groups VI-IX, while the antibody can be used to isolate the TNF receptor. The compound can be used in the method of Group IV. Therefore, the special technical features of the compounds of Groups I, II, and III distinguish one from the other.

The polynucleotides encoding TNF receptor and the TNF receptor of Group I are distinct from the methods of Groups IV and V because neither the polynucleotide or TNF receptor are used in these methods. Therefore, these Groups share no special technical feature.

The polynucleotides encoding TNF receptor and the TNF receptor of Group I are distinct from the methods of Groups VI-IX because the polynucleotides encoding TNF receptor and the TNF receptor can be used in any of the methods and therefore the polynucleotide and the TNF receptor is not considered to be special to any of these method.

The antibody of Group II is distinct from the methods of Groups IV-IX because the antibody is not used in any of these methods. Therefore, Group II and Groups IV-IX share no special technical feature and are distinguished from each other.

The compound of Group III is distinct from the method of Group IV because the compound can be used in another method such as to make antibodies and therefore its action at the TNF receptor is not considered to be special to the method.

The compound of Group III is distinct from the methods of Groups V-IX because the compound is not used in any of

# INTERNATIONAL SEARCH REPORT

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the methods. Therefore, Group III and Groups V-IX share no special technical feature and are distinguished from each other.

Groups IV-IX are distinct one from the other because the methods comprise differing steps, use of differing compounds, and attain differing goals and outcomes. Therefore, these Groups share no special technical feature and are distinguished from each other.